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(71)(72) Applicants and Inventors: JALKANEN, Sirpa [FI/FI]; Rauvalantic 112, FIN-20760 Piispaanristi (FI). SALMI, Marko [FI/FI]; Vähä-Hämeenkatu 12 a B 30, FIN-20500 Turku (FI).			Published <i>With international search report.</i>
(4) Agent: ORION CORPORATION; Orion-Farmos, Research & Development, Patent Service, P.O. Box 65, FIN-02101 Espoo (FI).			

(54) Title: COMPOSITIONS AND DIAGNOSTIC METHODS USING MONOCLONAL ANTIBODIES AGAINST CD44v6

(57) Abstract

Monoclonal antibody (DSM ACC1213) which binds only to forms of CD44 containing the amino acid sequence encoded by exon v6, but not to the 90 kilodalton standard lymphocyte form of CD44 and the use of such antibodies for detecting the presence of CD44v6 in tissues or cells, for determining if epithelial tissue from an animal or human has undergone malignant transformation, for determining the metastatic potential of malignant cells and for detecting inflammatory diseases in patients.

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COMPOSITIONS AND DIAGNOSTIC METHODS USING MONOCLONAL ANTIBODIES AGAINST CD44v6

The field of invention relates to immunological reagents and methods for detecting the expression of specific antigens. Specifically, the invention relates to a monoclonal antibody which detects a variant of the membrane glycoprotein CD44. The immunological reagent of the present invention is useful as a diagnostic tool for detecting malignant transformation, assessing metastatic potential and for diagnosing inflammatory diseases.

BACKGROUND OF THE INVENTION

Cell-cell and cell-matrix interactions are of fundamental importance to multicellular organisms in controlling growth, differentiation and in the migration of cells. CD44 is one of the molecules known to be involved in these adhesion-dependent processes. CD44 is a multifunctional glycoprotein involved in: lymphocyte-endothelial cell interactions; adhesion of cells to extracellular matrix proteins; lymphohemotopoiesis; homotypic adhesion; T cell activation and adherence; cytokine release; metastasis and the lateral movement of cells (1).

CD44 is widely distributed among several hematopoietic and non-hematopoietic cells including all subsets of leukocytes, erythrocytes, many types of epithelial cells, fibroblasts, smooth muscle cells and glial cells of the central nervous system (1). Most hematopoietic cells, fibroblasts and glial cells predominantly express a 90 kD form of CD44. Lymphocytes also express a 180 kD form which represents a chondroitin sulfate modification of the 90 kD backbone (2). In contrast, the CD44 antigen in epithelial cell lines is considerably larger (140-160 kD), and still larger forms, up to 230 kD have been described (3, 4, 5).

Recently, the molecular basis underlying the biochemically distinct forms of CD44 has been resolved. Molecular cloning of human CD44 from lymphoid lines revealed a gene which encodes an integral membrane glycoprotein having an N-terminal extracellular region, a short hydrophobic transmembrane region and a cytoplasmic tail (6, 7). Subsequently, the

structure of an epithelial form of 150 kD from keratinocytes and carcinoma cell lines was analyzed (3, 8). The epithelial form was found to contain an additional stretch of 132 amino acids inserted in the membrane proximal part of the peptide backbone common to both the lymphocyte and epithelial forms. Forms containing the same 132 amino acid sequence or a shorter part of it were also found in hematopoietic cells (9).

In both the rat, and in man, five distinct amino acid sequence elements (or "domains") have been identified which may be found expressed as part of the 90 kD core of CD44 protein (10, 11, 12, 13). These domains are encoded by 10 at least ten distinct exons named v1-v10 (14). CD44 molecules containing one or more of these exons within the common protein backbone are designated as variant forms to distinguish them from the major 90 kD lymphocyte form (standard). Herein, the term exon v6 will be used for nucleotides 1140-1267 of the largest known form of human CD44 (11).

15 CD44 isoforms play important and distinct roles in tumor invasiveness and metastasis. The standard 90 kD lymphocyte form apparently contributes to the metastatic capacity of non-Hodgkins lymphoma cells in man (15, 16). The lymphocyte form, but not the 150 kD epithelial form (containing exons v8-10 according to the exon nomenclature), also enhances local tumor 20 formation and the metastatic potential of transfected lymphoma cells in a nude mouse model (17). Expression of the epithelial form is increased in carcinoma cells lines, which may suggest a role in tissue invasiveness (7). Finally, using a monoclonal antibody which recognizes rat variant CD44, Herrlich et al. reported a direct correlation between CD44 expression and the 25 metastasis of adenocarcinoma cells in International Patent Application WO 91/17248; see also (10).

In man, analysis of the expression pattern of the variant forms of CD44 has been limited to studies on the CD44 variant mRNAs present in cells (11). This has been due to a lack of available monoclonal antibodies capable of 30 distinguishing between the standard and variant CD44s in man. There is now one report (18) on the expression of CD44 variant glycoprotein in human colorectal neoplasia and normal human tissue using polyclonal antibodies raised against a fusion protein that carries sequences of the human variant exon v6. The authors here show that normal colon epithelium was largely 35 negative, while all metastases were positive. An other paper (19) reports the production of monoclonal antibodies against the same fusion protein and the

use of them to study the expression of variant CD44 glycoprotein on human lymphoid cells and tissues, as well as on non-Hodgkins lymphomas. These studies confirm the earlier reports on upregulation of the expression of variant CD44 in connection to metastatic capacity.

5 European Patent Publication EP 538 754 discloses the surprising fact that monoclonal antibodies raised against rat variant CD44 have an immunosuppressive effect in rats.

BRIEF DESCRIPTION OF THE FIGURES

10 Figure 1. MAb Var3.1 is specific for human exon v6 of CD44. Binding of mAbs Var3.1, Hermes-3 and 3G6 (negative control) to v6 specific peptide and to an irrelevant peptide was determined. Results are presented as net absorbances (mean \pm SD) from triplicate samples of two independent ELISA experiments (net absorbance = absorbance to v6 specific peptide - absorbance to control peptide).

15 Figure 2 (A-C). Mab Var3.1 recognizes recombinant proteins carrying exon v6. A) Schematic representation of the PCR strategy used to amplify CD44v6 from HaCat cells (see text for details). Box represents the variant part of CD44 and exons v6 and v7 are highlighted as a darkened area. Numbering of nucleotides is based on data presented in Ref. 11.

20 B) Coomassie blue staining of the whole cell lysates of transformed bacteria after IPTG induction. Lane1: cells transformed with pGEX-2T (arrowhead: the -28 kD product of the parent vector); Lane 2: cells transformed with pGEX-2T-Var (arrow: the -60 kD fusion protein containing v6). C) Immunoblotting of the same lysates. Whole cell lysates of IPTG-induced bacteria transformed with pGEX-2T-Var (lanes 1 and 3), and with pGEX-2T (lanes 2 and 4) were stained with mAbs Var3.1 (lanes 1 and 2) and 3G6 (negative control, lanes 3 and 4). MAb Var3.1 stains the -60kD fusion protein (lane 1, arrow) but not the product of pGEX-2T (lane 2). Both mAbs non-specifically reacted with a -38 kD molecule. MW = molecular weight standards in kD.

25 Figure 3. Molecular weight of CD44v6. Hermes-3 reactive material was isolated from a leukopheresis sample using an affinity column, resolved in SDS-PAGE and blotted. Reactivity with mAbs Var3.1 and Hermes-3 was analyzed using immunoperoxidase method. Lane 1, Hermes-3; lane 2,

Var3.1; lane 3, 3G6 (negative control). Molecular weight standards (kD) are indicated on the right.

Figure 4 (A-E). Tissue distribution of v6 and Hermes-3 epitope containing forms of CD44 in man. (A) A tonsil section stained with mAb 5 Var3.1. Positive immunoperoxidase reaction is seen in the squamous cells of surface epithelium. Note the predominant staining in mid and upper layers. Lymphocytes are negative. (B) Higher magnification of tonsil epithelium stained with mAb Var3.1. (C) Expression of CD44v6 is heterogeneous on high endothelial venules. Some high endothelial venules 10 are brightly positive (black arrows), whereas others are negative or weakly positive (white arrow). (D) A parallel tonsil section stained with Hermes-3. This antibody also stains all the layers of surface epithelium, but the expression is most prominent in the basal layers. Lymphocytes in the lymphatic area are brightly positive. 15 (E) Hermes-3 epitope is absent from high endothelial venules (arrowheads pointing to the luminal surface). e, surface epithelium; la lymphoid area. Scale bar, 15 μ m.

Figure 5 (A and B). v6 is expressed on the surface of squamous epithelial cells. (A) The plasma membranes of the superficial cells in the 20 stratified squamous epithelium of tonsil are darkly stained by mAb Var3.1. Note that the more superficial side (arrows) of cells is systematically more strongly stained. Peroxidase reaction. (B) With gold-labeled secondary antibody the gold particles are localized along the plasma membrane. Streptavidin-gold without contrasting, the white outline (arrows) is the plasma 25 membrane.

Figure 6 (A and B). Expression of CD44v6 on blood lymphocytes. (A) Fresh PBL (PERM -, left column) do not express CD44v6 on their surface, but are Hermes-3 bright. When the cells are permeabilized with 1% 30 formaldehyde and acetone prior to staining (PERM +, right column), many cells become CD44v6 positive. X-axis is relative fluorescence on a log scale; Y-axis is cell number. (B) In immunofluorescence microscopy permeabilized PBL show intracellular staining for CD44v6, which preferentially is localized in the periphery of cells. Top: mAb 3G6, (negative control), middle: mAb Var3.1, bottom: mAb Hermes-3. Scale bar, 10 μ m.

35 Figure 7. v6-specific RNA is present in human PBL. RNA was isolated from PBL and HaCat cells, reverse transcribed to cDNA, PCR amplified with

primers B and C (see Fig. 2A), separated in agarose gel, transferred onto nylon membrane and hybridized with a v6 specific probe (probe G, Fig 2A). Lane 1: lymphocytes, lane 3 HaCaT cells. Lanes 2 (lymphocyte) and 4 (HaCaT) represents negative control reactions which were identical to those seen in lane 1 and 3 with the exception that no reverse transcriptase was added into the cDNA synthesis reaction.

Figure 8 (A-F). CD44v6 is associated with the cytoskeleton. Fixed and permeabilized HaCaT cells were treated without (A-B) or with (C-F) 0.5% NP-40 prior to immunofluorescence staining with mAb Var3.1 (A,C), Hermes-3 (B,D) and 3G6 (negative control; E,F). Significant amount of the Var3.1 reactive material was resistant to NP-40 treatment, while Hermes-3 staining was greatly diminished after the treatment. Scale bar, 10 μ m.

Figure 9 (A-H). Expression of CD44v6 in tumors. A benign cutaneous papilloma is positive with both mAb Var3.1 (A) and mAb Hermes-3 (B). A squamocellular carcinoma of the skin displays greatly diminished expression of CD44v6 (C), but remains brightly Hermes-3 positive (D). (E) Higher magnification from C. (F) Higher magnification from D. (G) Metastatic cells from squamocellular carcinoma are practically mAb Var3.1 negative, but (H) they still contain the Hermes-3 epitope. Scale bar 15 μ m.

Figure 10. Existence of CD44v6 in sera of patients suffering from chronic inflammation (rheumatoid arthritis). As representative examples, presence of CD44v6 and Hermes-3 epitope in serial dilutions (from 1:10 to 1:3200) of sera of two rheumatoid arthritis patients and of one normal subject are shown. Lanes A-C: normal subject; A: Hermes-3, B: Var3.1, C: 3G6, negative control. Lanes D-F: patient #1; D: Hermes-3, E: Var3.1, F: 3G6. Lanes G-H: patient #2; G: Var3.1, H: 3G6. Hermes-3 staining was negative. BSA, control wells containing 50 μ g BSA.

Figure 11. Nucleotide sequence of exon v6 of CD44. The nucleotides shown in bold type are those that encode aminoacids that were used to prepare the synthetic peptide used for immunization. Nucleotide numbering is based on data reported in Reference 11.

SUMMARY OF THE INVENTION

The present invention is directed to monoclonal antibodies capable of reacting with specificity to CD44 variants which contain the amino acid sequence encoded by exon v6.

5 The present invention is also directed to a method of detecting pathological conditions by determining whether specific variant forms of CD44 are being expressed in cells. In a preferred embodiment, the loss of expression of cell surface CD44v6 is used to detect the malignant transformation of cells, especially of squamous epithelial cells, as well as the 10 metastatic potential of those cells that have undergone malignant transformation. In a still more preferred embodiment, the reagent used to detect the loss of CD44v6 is a monoclonal antibody, Var3.1.

15 In another preferred embodiment, the invention is directed to the detection of inflammatory states in individuals by measuring CD44v6 in serum samples. Inflammatory diseases that can be detected using this method include rheumatoid arthritis and inflammatory bowel disease.

DETAILED DESCRIPTION OF THE INVENTIONDefinitions:

The following definitions are provided:

1. CD44: CD44 is a multifunctional glycoprotein found on the surface of a number of cells and involved in adhesion-dependent processes. The protein occurs in several different forms which are termed "isoforms". A 90 kD form is referred to as the standard or lymphocyte form. There is also an 150 kD isoform which is found in epithelial cells, among other forms.
2. CD44 variants: All forms of CD44 other than the 90 kD lymphocyte form are called variants. Variants contain the 90 kD lymphocyte CD44 protein, but have additional amino acid sequence elements ("domains") as well. The domains making up the CD44 variants are the result of the differential splicing of at least 10 exons comprising the CD44 gene.
3. CD44v6: Those variants of CD44 which contain within their primary structure the amino acid sequence encoded by exon 6 are termed "CD44v6".
4. Exon v6: Exon v6 is defined as nucleotides 1140-1267 of the CD44 gene of humans (Refs: 11 and 14). The sequence of exon v6 is shown in Figure 11.
5. Hermes-3: Hermes-3 is a monoclonal antibody recognizing a site on the 90 kD lymphocyte protein present in all forms of CD44.
6. Var3.1: Var3.1 is a monoclonal antibody deposited with the International Depositary Authority DSM, Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH at the address Mascherode Weg 1B, D-3300 Braunschweig, Germany. The deposit was made on June 4, 1993 and was given the accession number DSM ACC2131. The antibody is specific for those forms of CD44 containing the amino acid sequence encoded by exon v6.
7. The abbreviation "PBL" stands for "peripheral blood lymphocytes"; mAb stand for "monoclonal antibody"; FCS stand for "fetal calf serum"; and PBS stands for "phosphate buffered saline".

8. Inflammatory diseases: Inflammatory diseases are those diseases characterized by the cellular and histological reactions associated with inflammation. The typical signs of inflammation are redness; a localized feeling of warmth; swelling; pain; and sometimes, a loss of normal function.

5 9. Metastatic Potential: Metastatic potential is defined as the tendency of neoplastically transformed cells to migrate from one part of the body to another. Typically, metastatic potential will be expressed as the percentage of total tumor cells colonizing new sites.

10 10. Malignant transformation: Malignant transformation is the change of normal cells to cancerous cells. The main characteristics which indicate that a malignant transformation has occurred are uncontrolled cellular proliferation and a loss of differentiated function.

Monoclonal Antibody Specific for the Amino Acid Sequence Encoded by Exon v6

15 The present invention is directed to monoclonal antibodies which react specifically with those forms of CD44 that contain within their primary structure the amino acid sequence element encoded by exon v6 (Figure 11). Methods for producing monoclonal antibodies specific for particular antigens are well known in the art. A procedure adapted to the production of 20 monoclonal antibodies to the peptide encoded by CD44 exon v6 is described below in Example I. The antibody produced in the Example was given the designation Var3.1 and was deposited with the International Depositary Authority DSM Deutsche Sammlung Von Mikroorganismen Und Zellkulturen GmbH at the address Mascheroder Weg 1 B, D-3300 Braunschweig, 25 Germany. The deposit was made on June 4, 1993 and was given the accession number DSM ACC 2131.

30 The invention contemplates a variety of uses of exon v6-specific antibodies in general and of Var3.1 in particular. In all cases, detection of antibody-antigen complexes can be accomplished either using a labelled second antibody, i.e. an antibody recognizing an immunoglobulin chain present in the antibody binding to the exon v6-encoded antigen, or by labeling the first antibody directly. Examples of labels which are typically used for such purposes include radioactive labels (e.g. ^{125}I , ^{131}I , ^{14}C or ^3H); biotin; fluorescent labels (such as fluorescein, rhodamine or phycoerythrin) or 35 enzymes (such as horseradish peroxidase, alkaline phosphatase or urease).

Techniques for incorporating such labels into antibodies and for detecting labeled antibody-antigen complexes are well known in the art (see e.g. Hood *et al. Immunology*, 2nd edition, chapter 3 (1984)).

Monoclonal antibody Var3.1 can discriminate human CD44v6 from
5 those forms of CD44 that do not possess exon 6. The use of this and similarly
directed monoclonal antibodies for research purposes is encompassed by
the present invention and is illustrated below. The result of studies examining
the expression of CD44v6 in normal and diseased states led to the
conclusion that there is a correlation between the expression of this antigen
10 and malignant transformation, the metastatic potential of transformed cells
and the presence of inflammatory diseases in people.

CD44v6 Expression in Normal Cells

Studies using Var3.1 to examine antigen expression in normal cells
are described in detail in Example I and are summarized below. These
15 studies illustrate the way in which this antibody can be used for research
purposes. Moreover, the results from normal samples, when compared to the
results obtained in later studies using malignantly transformed samples or
samples obtained from patients with inflammatory diseases shows that the
expression of the CD44v6 antigen can be used for diagnostic purposes.

20 CD44v6 was found to be present in different types of epithelial cells,
dendritic cells and in the endothelial cells of blood vessels. Most abundant
expression was seen in squamous epithelial cells, where the Var3.1 epitope
appeared to be concentrated on the superficial side of the cells. Comparison
25 of the expression patterns between mAb Var3.1 and Hermes-3, an antibody
which binds to the constant part of the CD44 molecule, revealed several
interesting features (summarized in Table I).

At surface epithelia of tonsils, the reactivity of Var3.1 and Hermes-3
was different. Var3.1 stained the cells in the mid and upper layers of the
epithelium most intensely, whereas the basal layers displayed greatest
30 reactivity with mAb Hermes-3.

Connective tissue components were strongly reactive with Hermes-3
but did not stain with mAb Var3.1. High endothelium of blood vessels, on the
other hand, was Var3.1-positive, but Hermes-3-negative.

10

CD44v6 was not present on lymphoid cells of secondary lymphatic organs or on the surface of peripheral blood lymphocytes. All these leukocyte populations, however, stained brightly with mAb *Hermes-3*. Thus, the expression of exon 6 is restricted to a few specialized cell types, whereas

5 *Hermes-3* epitope is present on a wide variety of cells.

In contrast to the expression on the surface of epithelial cells *in vivo*, peripheral blood leukocytes and several epithelial cell lines only expressed CD44v6 intracellularly. It apparently distributed both as a membrane-associated form and diffusely in the cytoplasm. The acetone treatment *per se* (10 used for permeabilization) was not necessary for the accessibility of the Var3.1 epitope to mAb, since mAb Var3.1 stainings produced identical reaction patterns on acetone-fixed and non-fixed cryostat sections of tonsil. Therefore, acetone does not unmask the Var3.1 epitope by dissolving some lipid constituents of the cell membrane.

15 A considerable amount of CD44v6 was in NP-40 insoluble form, and thus, is most probably linked to cytoskeletal proteins. The cytoplasmic tail of the standard CD44 is known to be associated with ankyrin, which links transmembrane proteins to actin and fodrin in mouse T-lymphoma cells (26). CD44 also colocalizes with vimentin in WI-38 and with actin in 3T3 cells (27, 28).
20 Furthermore, A3D7 and *Hermes-1* (other anti-CD44 antibodies against the constant part of CD44) reactive material has been shown to exist in an NP-40 insoluble form in human T cells (29). The present results suggest that CD44v6 can, at least partly, account for these previously described detergent insoluble forms of CD44.

25 CD44v6 Expression Can be Used in the Diagnosis of Malignant Transformation and in Assessing the Metastatic Potential of Tumor Cells

The expression of CD44v6 was not altered in benign epithelial neoplasms. In contrast, malignant transformation was associated with the down-regulation of CD44v6. Moreover, the variant CD44v6 was practically absent from the metastatic cells. In contrast, the majority of malignancies remained *Hermes-3* positive. These observations held true in the material of 37 epithelial tumors studies (see Example I).

30 The result suggest that exon v6 is not responsible for the invasiveness or metastasis of epithelial squamous carcinomas. Rather, its expression seems to be associated with the regulated, normal differentiation and

proliferation of epithelial cells. Its expression is silenced during malignant transformation.

The above results are in agreement with results reported in the literature. In a recent study on the effects of standard and epithelial CD44 isoforms on tumor growth in an *in vivo* model, the standard 80-90kD form, but not the epithelial form, enhanced tumor invasiveness and metastatic activity (17). In studies of non-Hodgkin lymphomas, surface expression of Hermes-3 correlated positively to the prevalence of metastasis (15, 16). Since we were unable to detect exon v6 on the surface of any leukocyte subset or line, standard form is the most likely candidate in mediating the metastatic behavior of non-Hodgkin lymphomas as well.

Although the loss of CD44v6 expression is correlated with malignant transformation and increased metastatic potential in epithelial cells, other changes may take place when cells of a different origin undergo transformation. For example Günthert *et al.* found that expression of the variant form of CD44 in a rat carcinoma cell line resulted in the acquisition of metastatic properties (10). The expression of exon v6 was implicated as having a central role in this process.

It appears most likely, that the different cellular origin of the malignant material accounts for the differences observed. The studies on Var3.1 described above used carcinomas derived from human keratinocytes, whereas two adenocarcinoma cell lines with their derivatives/variants were studied in the rat model (10). Preliminary studies in human samples using Var3.1 on adenocarcinoma specimens indicate that, in certain cases, exon v6 is up-regulated during malignant transformation. In a recent paper, it was reported that several alternatively-spliced large molecular weight variants were overproduced in malignant tumors of breast and colonic tissue in man when analyzed by PCR and hybridization (24). Thus, the role of CD44 in tumor metastasis may be dependent on the species, type of carcinoma or host microenvironments.

In terms of the invention as claimed herein, the result indicate that monoclonal antibodies specific for an epitope encoded by exon v6 can be used to detect malignant transformation and metastatic potential. In the case of malignant transformation, samples of the tissue suspected of being malignant would be obtained using standard biopsy techniques. Monoclonal antibody would then be used to compare the amount of CD44v6 present in

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the biopsy samples with the amount present in reference samples taken from the same type of tissue but from individuals known to be normal. The exact procedure used for the assay could follow the immunohistochemical procedures described in Example I or could take the form of other standard diagnostic immunoassays. In tissues of epithelial origin, malignant transformation should be accompanied by a loss of exon v6 antigen relative to normal tissue.

Assays performed for the purpose of evaluating the metastatic potential of cell would be similar to those for detecting malignant transformation except that comparison would be between a biopsy sample and cells known to be malignantly transformed but non-metastatic. Again, sample and reference tissue should be matched according to organ type, e.g. liver samples should be compared with references of liver origin. In the case of tissues of squamous epithelial origin, increased metastatic potential should be inversely correlated with the presence of the CD44v6 antigen.

CD44v6 Expression Can be Used in the Diagnosis of Inflammatory Diseases

Serum samples from normal individuals and from individuals suffering from chronic inflammatory diseases (either rheumatoid arthritis or inflammatory bowel disease) were collected and examined for the presence of CD44v6 antigen using the Var3.1 antibody. Assays performed using the dot blot procedure described in Example I indicated that samples from patients with inflammatory diseases showed strong reactivity with the antibody whereas samples obtained from normal individuals showed no or weak reactivity.

These results indicate that the CD44v6 epitope can be used for the diagnosis of inflammatory diseases. Serum samples from patients suspected of having inflammatory bowel disease would be compared to serum samples from normal individuals. The exact procedure could follow the dot blot procedure set forth in Example I or any other commonly used immunoassay procedure. The presence of inflammatory disease would be detected as an increase in antibody reactivity in the collected samples relative to the normal reference samples.

Having now generally described this invention, the same will be further described by reference to certain specific examples which are provided herein purposes of illustration only and are not intended to be limiting unless otherwise specified.

5

EXAMPLE I

Production of mAb against human exon v6 of variant CD44

Exon v6 has been reported to play a crucial role in the development of metastatic deposits of rat adenocarcinoma cells (10). To study the expression of CD44v6 in man, mAbs against a synthetic polypeptide from exon v6 were 10 produced.

A synthetic peptide representing a 16 amino acid sequence from the exon v6 of the human variant CD44 (STTEETATQKEQWFGN (11); an additional C-terminal cysteine was included for coupling purposes) was prepared using an automated peptide synthesizer (Model 431 A, Applied 15 Biosystems, CA). Purification of the peptide was carried out with a preparative HPLC (Applied Biosystems) using a reverse phase column and its purity was confirmed by an analytical HPLC. The peptide was also independently sequenced (Model 477A equipped with an online PTH amino acid analyzer 120A, Applied Biosystems) and found to be correct. One 20 hundred microgram peptide in incomplete Freund's adjuvant was injected into the footpads of specific pathogen free Balb/c mice three times at one week intervals. After sacrifice lymphocytes from popliteal lymph nodes were isolated and fused with NS-1 myeloma cells using standard 25 procedures. Hybridoma supernatants were tested in ELISA (see below) using the synthetic peptide as an antigen.

Production of anti-CD44 mAbs of Hermes-series has been described earlier (21). Hermes-3 recognizes an epitope in the proximal extracellular part of the constant region of CD44 (6). 3G6, a mouse mAb against chicken T cells, was used as a negative control. All antibodies were used as serum-free 30 supernatants or as (NH₄)₂SO₄ precipitated concentrates.

The hybridomas were screened by using ELISA: The synthetic peptide from the exon v6 and a control peptide (DELPQVTLPHPNLHGPEILDVPST) were absorbed to the bottom of microtiter wells (Dynatech Laboratories, Alexandria, VA) overnight at 37°C

(10µg/well). After washings, the remaining binding sites were blocked with 1% gelatin, and after washings the primary antibodies were added for 2h. Alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (Tago, Burlingame, CA) was used as the second stage antibody, and p-nitrophenylphosphate as the substrate. Absorbances were read in Multiscan (Labsystems, Helsinki, Finland) at 405 nm.

5 Supernatant from one hybridoma specifically recognized the peptide used for immunization (Fig 1). This exon v6 specific mAb (designated mAb Var3.1) was selected for further studies after subcloning twice by limiting 10 dilution. The isotype of mAb Var3.1 was Ig G₁.

EXAMPLE II

Preparation and purification of antigen proteins containing exon v6 of human variant CD44

Exon v6 containing form of CD44 was amplified from HaCaT cells by 15 reverse transcriptase polymerase chain reaction (PCR) and cloned into pGEX-2T vector (22) for fusion protein pruduction. The PCR-primers were as follows:

A: 5' CAATTACCATAACTATTGTTAACCG 3'
B: 5' AATCAGTCCAGGAACTGTCCT 3'
20 C: 5' GGCAACAGATGGCATGAGGG 3'
D: 5' AGTGGTATGGGACCCCCCACTGGG 3'
E: 5' ATAGGATCCAACCGTGATGGCACCCGCT 3'
F: 5' TATGAATTCGGAATGTGTCTTGGTCTC 3'
G: 5' GCTGTCCCTGTTGTCGAATG 3'

25 HaCaT RNA was isolated by the guanidine isothiocyanate - phenol extraction method. First strand cDNA synthesis was carried out using 1.5 µg total RNA, oligo(dT) primer and M-MLV reverse transcriptase according to the instructions of the manufacturer (Perkin Elmer Cetus, Norwalk, CT). Bacause HaCaT cells have several forms of CD44 (unpublished PCR data, K.G-V., 30 M.S., S.J.), existence of v6 in the PCR product was ensured by using two sets of primers in two separate PCR amplification reactions as schematically illustrated in Figure 2A. In one reaction (Ia), primers A and B were used and in the other reaction (Ib), primers C and D were employed. Thereafter, the products were combined in a reaction using primers E and F which contained 35 BamH1 and EcoR1 tails, respectively. The presence of exon v6 in the 0.95 kb

PCR product was confirmed by dot blot hybridization with the P^{32} -labelled probe G and by sequencing.

The 0.95 kb fragment (including exons v6-10) was isolated from 1.5 % agarose gel, digested with BarnH1 and Eco R1 and ligated using T4 ligase (Stratagene) into pGEX-2T expression vector (23, 22). Hereafter, the plasmid pGEX-2T with the 0.95 kb v6 containing insert is called pGEX-2T-Var. Bacteria (*E. coli* DH5 α strain) were transformed with pGEX-2T-Var using CaCl₂ method. Production of fusion protein was induced with isopropyl β -D-thiogalactopyranoside (IPTG), after which bacteria were pelleted and lysed in Laemmli's sample buffer containing 2 % SDS.

Peripheral blood cells from leukopheresis samples of patients suffering from rheumatoid arthritis were used for CD44 antigen isolation as previously described (20). Briefly, lymphocytes (25 ml packed cells) were lysed in lysis buffer (1 % NP-40, 0.15 M NaCl, 0.01 M Tris, 1.5 mM MgCl₂ and 1 mM PMSF, pH 7.0). The clarified lysate was applied first to a Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column and then sequentially to three CNBr-activated Sepharose-4B (Pharmacia) columns derivatized with normal mouse serum, with irrelevant mAb and with Hermes-3 mAb (5 mg/ml, 3 ml column volume). The column was washed extensively with the lysis buffer. Thereafter, the material bound to the Hermes-3 column was eluted with 50 mM triethylamine and lyophilized. Isolated D44 was subjected to SDS-PAGE and blotting as described below for fusion proteins with the exception that membranes were blocked in 0.1 % Tween-20 for 3h and 5 % AB-serum (Finnish Red Cross, Helsinki, Finland) was added with the second-stage antibody.

Total RNA was isolated from PBL obtained from blood donors and from HaCaT cells using guanidine isothiocyanate method and reverse transcribed to cDNA. To study the presence of exon v6 containing mRNA in these cells, primers B and C (Fig. 2A) were used for PCR. The PCR products were separated in 1.5 % agarose gel, blotted onto nylon membranes (Zeta-Probe, BioRad), hybridized to a P^{32} -labeled oligonucleotide probe from exon v6 (probe G in Fig 2A) according to standard procedures for Southern blotting (23). As controls, parallel reactions were performed that contained all the reagents except reverse transcriptase.

EXAMPLE IIISpecificity of mAb Var3.1

The specificity of mAb Var3.1 was demonstrated by showing that it reacted with the recombinant protein containig human v6 according to

5 Example II. Samples from whole cell lysates of pGEX-2T-Var and pGEX-2T (control) transformants were run on a 5-12.5 % SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Schleicher-Schuell, Dassel, Germany) by blotting 1.5 h at 1A in a Transphor apparatus (Hoefer, San Francisco, CA). The membranes were then soaked

10 for 48 h in PBS containing 0.1 % Tween-20 and 1 % non-fat milk powder. After washings in PBS, membranes were cut into strips and incubated overnight with primary antibodies. After washing, a 3h incubation in PBS containing peroxidase-conjugated rabbit anti-mouse Ig (Dakopatt A/S, Glostrup, Denmark) was performed. After extensive washing, the membranes

15 were developed in PBS containing 16 % methanol, 0.5 mg/ml 4-chloro-1-naphthol (Sigma) and 0.01 % hydrogen peroxide. Same amount of samples were also run on parallel gels which were subsequently fixed in 40 % methanol and 10 % acetic acid and stained with Coomassie Brilliant blue (Fig 2B).

20 It was found that mAb Var3.1 reacted with the ≈60 kD fusion protein but not with the product of the parent vector alone. A negative control mAb failed to stain the ≈60 kD molecule. Together, the results of ELISA and fusion protein assays unambiguously show that mAb Var3.1 recognizes v6 of CD44 in man.

25 Immunoblotting analyses of Hermes-3 purified CD44 antigen from leukopheresis samples (See Example II) revealed that under nonreducing conditions mAb Var3.1 recognized two major bands (≈220 and 300 kD), and one faint bigger band (Fig. 3). This experiment shows that mAb Var3. 1 recognizes an epitope of purified CD44. MAb Hermes-3 stained proteins of

30 very variable sizes (70-300 kD) from the purified CD44 material (Fig. 3).

EXAMPLE IVExpression of CD44v6 in normal tissues

Tissue distribution of the different forms of CD44 was determined using immunoperoxidase staining. Surgical and skin punch biopsy specimens
5 were snap frozen in liquid nitrogen. Five μ m frozen sections were cut, air-dried and acetone fixed. Sections were overlaid with mAb supernatants and incubated for 30 min at room temperature in a humidified chamber. After two washings in PBS, peroxidase-conjugated rabbit anti-mouse Ig in PBS containing 5% AB-serum was added. Finally, the reaction was developed by
10 adding 3,3-diaminobenzidine (Polysciences, Inc., Warrington, PA) in PBS containing 0.03% hydrogen peroxide for 3 min. After staining, the sections were counterstained in hematoxylin (Sigma Chemicals, St. Louis, MO), dehydrated, cleared in xylene and permanently mounted in DePex (BDH Limited, Pool, Dorset, England).

15 For immunoelectronmicroscopy, samples from human tonsils were snap-frozen in freon 22 chilled with liquid nitrogen. About 15 μ m frozen sections were stained either immediately or after short fixation in -20°C acetone. The immunoperoxidase staining was performed as described above. The reactions were followed by fixation in phosphate buffered 2%
20 glutaraldehyde. Thereafter, a representative section was examined light microscopically, and an appropriate area was selected. The corresponding area in parallel sections was trimmed, sections were postfixed in phosphate buffered 2% osmium tetroxide, dehydrated and embedded in epon at the open end of an inverted BEEM capsule. Alternatively, gold labeling was
25 done by sequential incubations with mAb Var3.1, biotinylated horse anti-mouse Ig (Vector laboratories, Burlingame, CA), and aggregated streptavidin-gold solution (Zymed, San Francisco, CA). Slides were processed similarly as described for immunoperoxidase reaction, except that silver intensification was used for visualization of the reaction product, and
30 osmium tetroxide fixation was omitted. Thin sections were double-stained with uranyl acetate and lead citrate and then examined in a JEM 100 electron microscope. Slides processed without the primary or secondary antibody, with and without the double staining served as controls.

In the tonsil, surface epithelium intensely stained with mAb Var3.1 (Fig
35 4 a and b). Reactivity was particularly strong in the mid and upper layers of

stratified squamous epithelium (upper stratum spinosum and stratum granulosum), while cells in the basal layers exhibited fainter staining. Reticulated crypt epithelium also stained positively with mAb Var3.1. Tonsillar lymphocytes did not react with mAb Var3.1, and connective tissue components were also negative (Fig 4 a). In germinal centers, mAb Var3.1 faintly reacted with cells of dendritic morphology. Luminal surface of some blood vessels, including high endothelial venules, also stained with mAb Var3.1 (Fig 4 c). In comparison, expression of the Hermes-3 epitope on tonsillar surface epithelium was most pronounced basally and notably less was seen in upper layers (Fig 4 d). MAb Hermes-3 intensely reacted with practically all lymphocytes outside the germinal centers, and fibroblasts were strongly positive in the septae. On the other hand, endothelial lining of most vessels and all high endothelial venules was Hermes-3 negative (Fig 4 e).

In electron microscopy the plasma membrane of the superficial squamous cells stained homogeneously dark in the peroxidase preparations, and the staining was usually more intense on the superficial side of the cell (Fig 5 a). In gold preparations the particles were localized along the surface of the superficial squamous cells (Fig 5 b).

TABLE 1

Cell and Tissue Distribution of VAR3.1 and Hermes-3 epitopes of CD44

	Var3.1	Hermes-3
Blood cells (surface)		
PBL	-	++
granulocytes	-	++
monocytes	-	++
Tonsil		
lymphocytes	-	++
dendritic-like cells in germinal centers	+	+
HEV	+	+
surface epithelium	++	++
Skin		
stratum basale	+	++
stratum spinosum	+	++
stratum granulosum	++	+
dermal fibroblasts	-	+
hair follicles	++	++
sweat glands	+	++
Intestine		
lymphoid cells	-	++
enterocytes	-/+	++
smooth muscle	-	++
Brain		
neurons	-	-
glial cells	-	++

5 Blood cells were surface stained for immunofluorescence and analyzed with FACS. Frozen sections of tissues were stained using immunoperoxidase staining. Intensity of staining was scored as follows: -: negative; -/+: weak, +: moderate, ++: strong. HEV= high endothelial venules.

10 Expression patterns obtained by mAbs Var3.1 and Hermes-3 were clearly distinct in several other tissues in addition to tonsil. In the skin, CD44v6 was preferentially localized in the upper layers of the epidermis, except in keratinized surface layer that was negative (data not shown). In the dermis, fibroblasts and other stromal elements were negative, while hair follicles and sweat glands stained positively with mAb Var3.1. Expression of
15 the Hermes-3 epitope in the epidermis was most prominent basally, and in

20

the dermis, Hermes-3 epitope was abundantly present in fibroblasts. In the intestine, enterocytes stained positively with mAb Var3.1. Weaker reactivity was observed in dendritic cells of Peyer's patches, whereas CD44v6 was absent from other structures. MAb Hermes-3 stained enterocytes more

5 strongly, and the Hermes-3 epitope was also present in lymphoid, smooth muscle and connective tissue cells of the gut. CD44v6 was not present on neurons or glial cells of brain white or gray matter. Peripheral nerves also lacked this molecule. In contrast, glial cells of white matter were intensely positive with mAb Hermes-3.

10

EXAMPLE V

Intracellular localization of CD44v6 in blood leukocytes and cell lines

Human PBL were isolated from healthy adult volunteers and from patients suffering from chronic inflammatory diseases (severe rheumatoid arthritis or inflammatory bowel diseases) using Ficoll-gradient (Ficoll-15 Hypaque, Pharmacia) centrifugation. PBL were used fresh or, to obtain activated blast cells, stimulated with combination of PHA and PWM (Gibco, Grand Island, NY) for 3 days at 37°C in RPMI 1640 (Gibco) supplemented with 10% FCS, glutamine, Hepes, sodium pyruvate, penicillin and streptomycin.

20 Human cell lines HeLa (epithelioid carcinoma), KG-1, KG-1a, K-562 (leukemic cells), and A549 (lung carcinoma) were obtained from American Type Culture Collection (Rockville, MD). U1690 (human lung carcinoma cell line) was a kind gift from Dr. H. Hirvonen (Department of Medical Biochemistry, Turku University, Finland) and HaCaT, a spontaneously immortalized, nontumorigenic keratinocyte line, was a kind gift from Prof. N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany). All adherent cell lines were cultured in Dulbecco's modified minimal essential medium (Gibco) supplemented with 10% human AB-serum, 10 mM Hepes and antibiotics and passaged using trypsin-EDTA (Boehringer-Mannheim, 25 Germany).

30 Blood cells and adherent cell lines (detached with 5 mM EDTA in Ca²⁺, Mg²⁺-free HBSS) were stained in suspension. Cells were stained unfixed or after fixation and permeabilization (1% formaldehyde in PBS (10 min) followed by ice-cold acetone (5 min) followed by two washings in PBS). 35 Cells were incubated with primary antibodies for 20 min at 4°C and washed

twice in PBS containing 5% FCS and 1mM sodium azide. Next, FITC-conjugated sheep anti-mouse IgG (Sigma) in PBS containing 5% human AB-serum was added for 20 min. Thereafter, the cells were washed twice and fixed in PBS containing 1% formaldehyde. Analyses were done using a

5 FACScan cytometer (Becton Dickinson, Mountain View, CA). For immunofluorescence microscopy cells were spun on microscopic slides with Cytospin 2 cytocentrifuge (Shandon Southern, Surrey, England) and mounted in glycerol containing 10 % PBS. Alternatively, adherent cells were grown on glass coverslips. Thereafter, they were processed without

10 detachment for immunofluorescence microscopy as described above.

CD44v6 was absent from the surface of PBL, monocytes and granulocytes as determined by FACS-analysis (Fig 6). PWM/PHA-induced immunoblasts and plasma cells did not express this form of CD44 either. Also, *in vivo* activated PBL (chronic inflammation) were surface-negative with

15 mAb Var3.1. However, when the cells were subjected to permeabilization prior to the immunofluorescence staining, majority of both unactivated and activated lymphocytes expressed Var3.1 epitope (Fig 6). Number of positive cells ranged between 50 and 100% in different individuals. In fluorescence microscopy, positive reactivity was preferentially localized in the periphery of

20 the cells, and fainter diffuse staining was detectable throughout the cytoplasm (Fig 6 *b*). CD44v6 was also present at RNA level in lymphocytes (Fig 7). In contrast, the Hermes-3 epitope was abundantly expressed on surfaces of all blood leukocyte subtypes as confirmed in FACS and immunofluorescence microscopy analyses (Fig 6).

25 When studying the detergent resistance of Var3.1 and Hermes-3 epitopes, HaCaT cells grown on glass slides and cytocentrifuge preparations of PBL were used. Cells were first fixed and permeabilized as described above. Thereafter, cells were incubated in PBS with or without 0.5% NP-40 for 5 min at 4°C and washed twice. Next, cells were stained for

30 immunofluorescence as described above, and analyzed using fluorescence microscopy. Similar results were obtained, when the cells were first stained and thereafter treated with the detergent.

Since CD44 is known to associate with cytoskeleton, we determined whether CD44v6 would also be linked to cytoskeletal proteins.

35 Permeabilized HaCat cells were incubated in PBS with or without 0.5% nonionic detergent NP-40 and stained for immunofluorescence (Fig. 8).

Immunofluorescence microscopy showed that a considerable amount of CD44v6 was in NP-40 insoluble form. In contrast, significant amount of of Hermes-3 containint form of CD44 disappeared during the NP-40 treatment. Similar results were obtained when PBL were analyzed (data not shown).

5 No molecular mass for CD44v6 was obtained from the NP-40 lysates of HaCaT cells in Western blotting or in immunoprecipitations after labelling with 35C-Methionine, 35S-Cystein, 35Sulphate and 14C-Glucosamine, probably due to the poor NP-40 solubility of CD44v6 in these cells. Moreover, mAb Var3.1 aslo appears to be a poorly precipitating antibody.

10 However, in immunoblotting of SDS solubilized HaCaT cells, a faint ≈200 kD band was seen (data not shown).

Human keratinocyte (HaCaT), epithelial carcinoma (HeLa, U1690, A549) and hematopoietic (KG-1, KG-1a, K562) cell lines all lacked CD44v6 on their surface. However, all of them tested after permeabilization (HaCaT, 15 HeLa, U1690) showed clear intracellular staining with mAb Var3.1. In contrast to CD44v6, all cell lines displayed Hermes-3 reactivity both on the cell surface and in the cytoplasm (data not shown).

EXAMPLE VI

Downregulation of CD44v6 in human neoplasms

20 The role of different CD44 forms in spread of malignancies is currently under dispute. Therefore, we stained 37 samples from benign (7 papillomas) and malignant (total 30: 5 metastatic, 5 grade III, 10 grade II and 10 grade I head and neck squamous cell carcinomas) epidermal tumors for expression of CD44v6. These experiments showed that all epidermal cells in benign 25 neoplasms stained with mAb Var3.1 like their normal counterparts in the neighboring healthy tissue. Surprisingly, expression of CD44v6 was down-regulated in all carcinoma samples in the malignant areas. In general, better differentiated carcinomas displayed more intense mAb Var3.1 reactivity than the more undifferentiated ones. As examples, staining patterns of a benign 30 papilloma and a squamocellular carcinoma are shown in Figure 9. All distant metastatic lesions of squamocellular carcinomas were practically negative with mAb Var3.1. In contrast, Hermes-3 brightly stained all benign and majority of the malignant cell types, including the metastatic deposits, in these specimens (Fig 9).

EXAMPLE VIICD44v6 levels in serum in patients with inflammatory diseases

Dilution series from serum samples of normal subjects and from patients suffering from chronic inflammatory disorders were analyzed using 5 the dot blot assay. Serial dilutions of serum samples or BSA (50 µg/well as control) were transferred onto nitrocellulose membrane (Schleicher-Schuell) using a dot blot apparatus. The membrane was blocked in 1% non-fat milk for 2 h at room temperature and then washed twice in PBS. Thereafter, the membrane was cut into strips and the strips were stained as described for 10 Western blottings.

Normal sera showed no positive reactivity with MAbs VAr3.1. In contrast, samples from chronically ill patients contained material that strongly stained with CD44v6 specific MAbs (Fig. 10). Hermes-3 reactive material was not detected using this method.

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- 25 -

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>7</u> , line s <u>21 - 27</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1 B D-38124 Braunschweig GERMANY	
Date of deposit	Accession Number
1993-06-04	DSM ACC2131
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
For receiving Office use only	
<input checked="" type="checkbox"/> This sheet was received with the international application	
For International Bureau use only	
<input type="checkbox"/> This sheet was received by the International Bureau on:	
Authorized officer	
	

CLAIMS

1. A monoclonal antibody which binds to forms of CD44 containing the amino acid sequence encoded by exon v6, but not to the 90 kilodalton standard lymphocyte form of CD44, wherein said monoclonal antibody is 5 Var3.1, deposited with the International Depositary Authority DSM, Deutsche Sammlung Von Mikroorganismen Und Zellkulturen GmbH as accession no. ACC2131.
2. A method for detecting the presence of CD44v6 in tissues or cells, 10 wherein said tissues or cells are reacted with the antibody according to claim 1.
3. The method according to claim 2, wherein said tissues or cells are of 15 human origin.
4. A method for determining if a tissue from an animal or human has undergone malignant transformation, characterised by
 - a) obtaining the tissue sample to be assayed by biopsy or surgery;
 - b) determining the amount of CD44v6 present in said tissue by reacting 20 the tissue sample with the monoclonal antibody which binds to forms of CD44 containing the amino acid sequence encoded by exon v6, but not to the 90 kilodalton standard lymphocyte form of CD44;
 - c) comparing the results obtained in step b) with the results from similar reactions carried out using reference samples wherein said reference 25 samples are known to be normal;
 - d) identifying transformed tissues as those where the difference between the CD44v6 levels in the tissue sample is statistically significantly decreased relative to reference samples.
5. A method according to claim 4, wherein said tissue is squamous epithelial tissue. 30
6. The method for determining the metastatic potential of malignant cells, characterized by
 - a) obtaining the tumor sample to be assayed by biopsy or surgery; 35

- b) determining the amount of CD44v6 present in said tumor sample by reacting the tumor sample with the monoclonal antibody of claim 1;
- c) comparing the results obtained in step b) with the results from similar reactions carried out using reference samples wherein said reference samples are tumor cells of the same type as the tumor sample, but which are known to be non-metastatic;
- 5 d) identifying tumors of high metastatic potential as those where the difference between the CD44v6 levels in the tissue sample and the reference sample is statistically significant.

10

7. The method according to claim 6, wherein the tumor sample is of epithelial origin and wherein high metastatic potential is indicated by a statistically significant decrease in CD44v6 relative to the reference samples.

15

8. A method for detecting inflammatory diseases in patients, characterized by

- a) obtaining a serum sample from said patient;
- b) determining the amount of CD44v6 present by reacting the sample with a monoclonal antibody which binds to forms of CD44 containing the 20 amino acid sequence encoded by exon v6, but not to the 90 kilodalton standard lymphocyte form of CD44;
- c) comparing the results obtained in step b) with the results from similar reactions carried out using serum samples from normal individuals;
- d) identifying those individuals with inflammatory diseases as those 25 whose serum samples have a statistically significant increase in CD44v6 relative to the amount of CD44v6 in the serum samples from normal individuals.

30

9. A method according to claim 8, wherein the monoclonal antibody is an antibody according to claim 1.

35

10. The method according to claims 8-9, wherein said inflammatory diseases are rheumatoid arthritis or inflammatory bowel disease.

11. The method according to claims 2-10, wherein the amount of CD44v6 is determined by means of a dot blot assay.

12. The method according to claims 2 - 10, wherein the presence of CD44v6 is detected using immunoperoxidase staining.

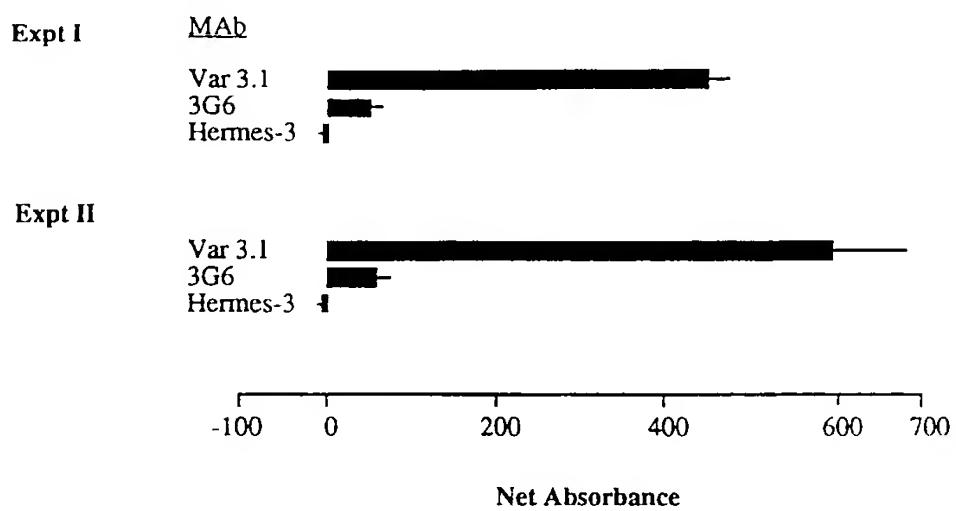


Fig. 1

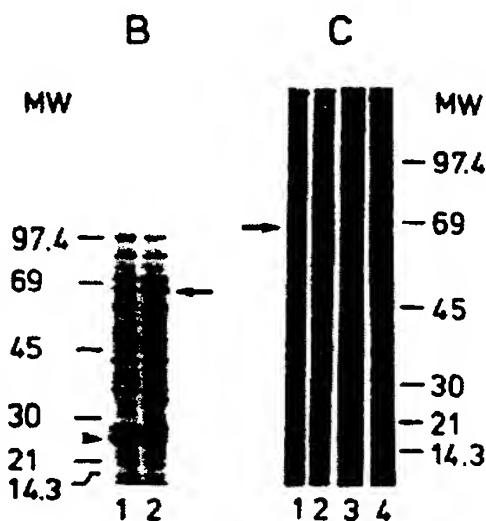
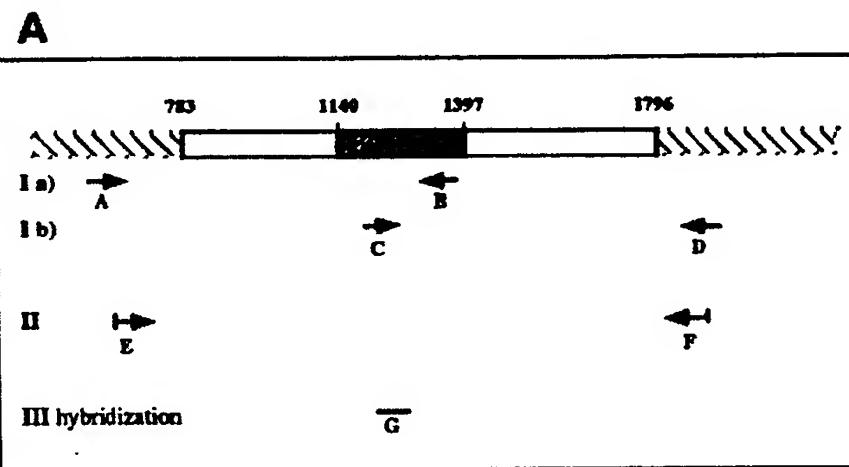


Fig. 2

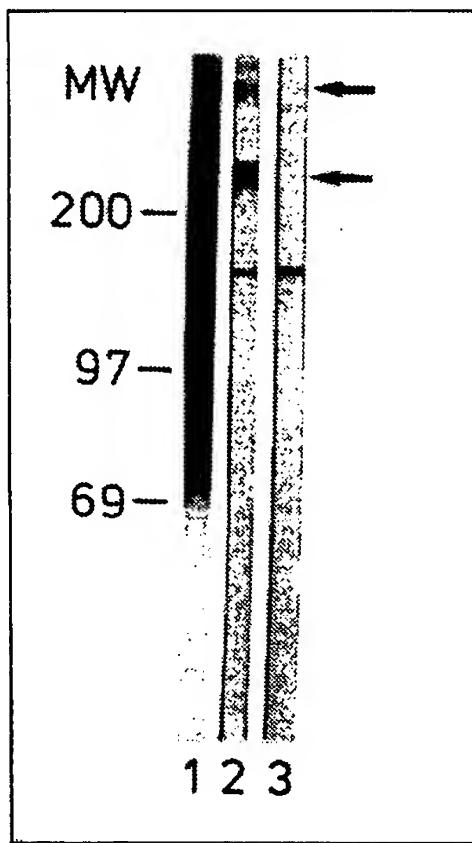


Fig. 3

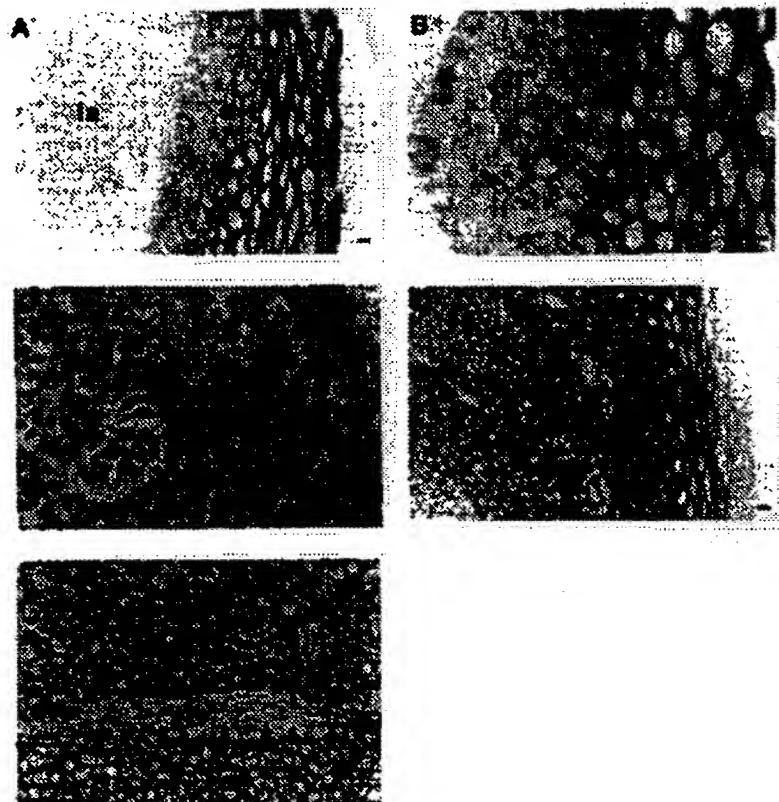


Fig. 4

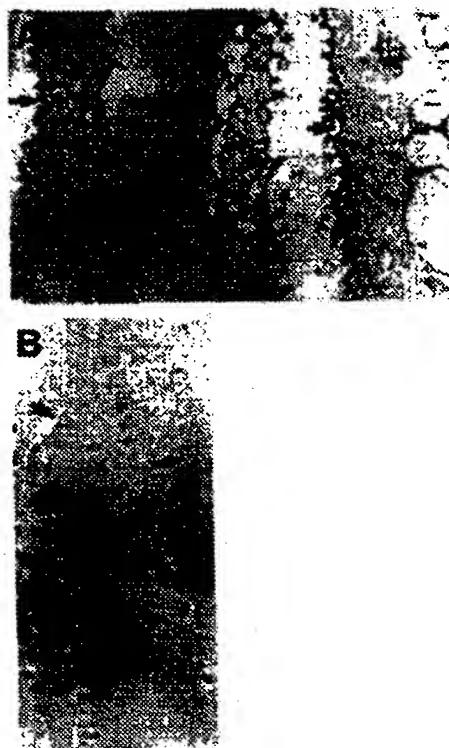


Fig. 5

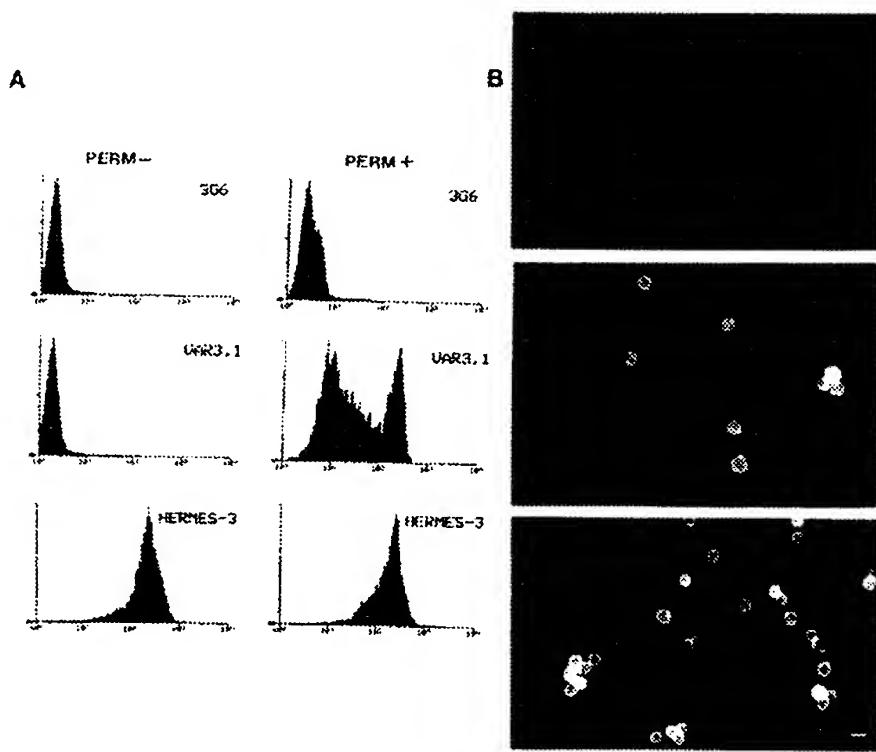


Fig. 6

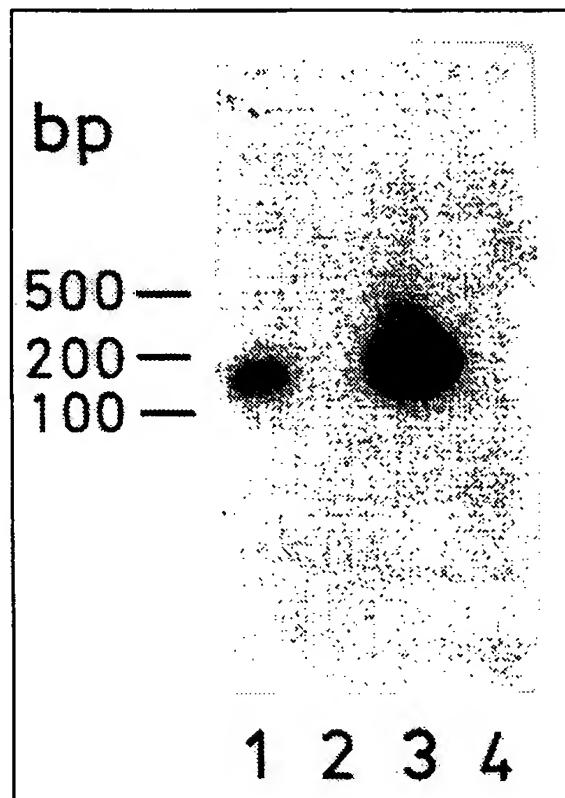


Fig. 7

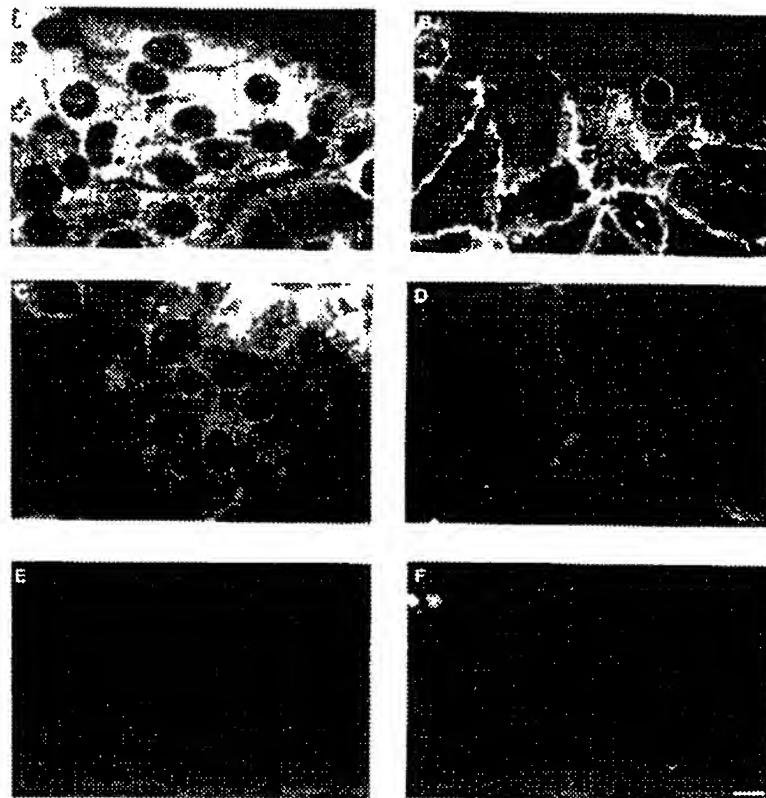


Fig. 8

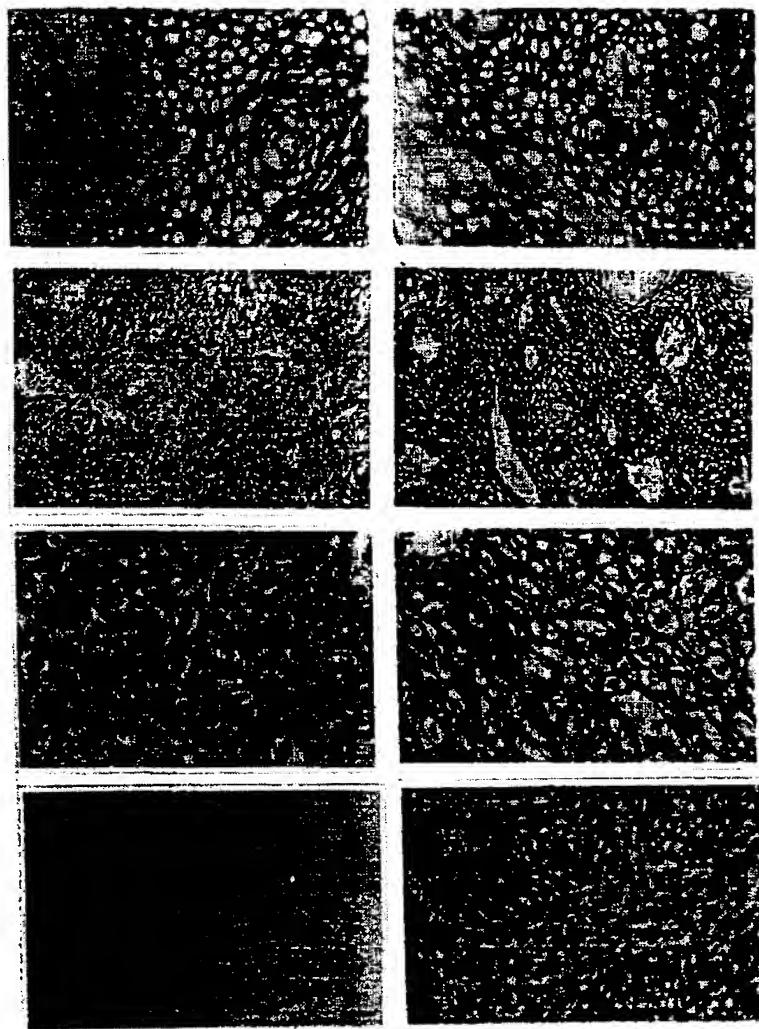


Fig. 9

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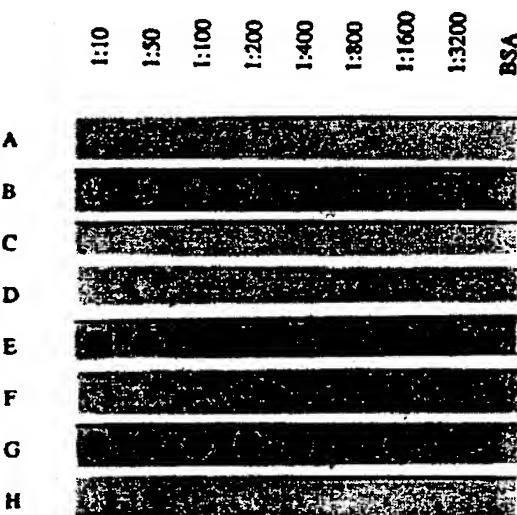


Fig.10

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Pos. 1140

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AGA TGG CAT GAG GGA TAT CGC CAA ACA CCC AGA
GAA GAC TCC CAT TCG ACA ACA GGG ACA GCT G-

Pos. 1267

Fig. 11

Applicant's or agent's file
reference number

CD44/SN

International application N
PCT/FI94/00264

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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A. The indications made below relate to the microorganism referred to in the description
on page 7, line 21 - 27

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN
UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1 B
D-38124 Braunschweig
GERMANY

Date of deposit

1993-06-04

Accession Number

DSM ACC2131

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Applicants request that, until the application has been laid open to public inspection by the national offices, or has been finally decided upon, the furnishing of a sample shall only be effected to an expert in the art.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

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BU...PEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
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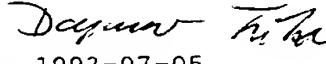
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Dr. Sirpa Jalkanen
Dr. Marko Salmi
National Public
Health Institute
Kiinamyllynkatu 13
20520 Turku
Finland

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITORY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR		II. IDENTIFICATION OF THE MICROORGANISM
Name: Dr. Sirpa Jalkanen Dr. Marko Salmi Address: National Public Health Institute Kiinamyllynkatu 13 20520 Turku Finland		Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: DSM ACC2131 Date of the deposit or of the transfer ¹ : 1993-06-04
III. VIABILITY STATEMENT		
The viability of the microorganism identified under II above was tested on 1993-06-07 ² On that date, the said microorganism was (<input checked="" type="checkbox"/>) ³ viable (<input type="checkbox"/>) ³ no longer viable		
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴		
IV. INTERNATIONAL DEPOSITORY AUTHORITY		
Name: DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1 B D-38124 Braunschweig		Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 1993-07-05

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

4 Fill in if the information has been requested and if the results of the test were negative.

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FOR THE PURPOSES OF PATENT PROCEDURE

PCT/FI 94 / 00264

INTERNATIONAL FORM

Dr. Sirpa Jalkanen
Dr. Marko Salmi
National Public
Health Institute
Kiinamyllynkatu 13
20520 Turku
Finland

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITORY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY:
Var 3.1	DSM ACC2131
II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: (<input checked="" type="checkbox"/>) a scientific description (<input checked="" type="checkbox"/>) a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depository Authority accepts this microorganism identified under I. above, which was received by it on 1993-06-04 (Date of original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I. above was received by this International Depository Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Adress: Mascheroder Weg 1 B D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized official(s):  Date: 1993-07-05

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired

INTERNATIONAL SEARCH REPORT

Int. Search Report No
PCT/FI 94/00264A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12P21/08 G01N33/577 G01N33/574

According to international Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	'8th INTERNATIONAL CONGRESS OF IMMUNOLOGY, Budapest, Hungary, August 23-28, 1992' August 1992, SPRINGER VERLAG, BUDAPEST, HUNGARY see M. SALMI et al.: 'Expression of domain 3 containing isoforms of CD44 in man. (page 274, abstract W-47/I-34) ---	1-7, 11, 12
A	INTERNATIONAL JOURNAL OF CANCER vol. 46, no. 5, 15 November 1990, GENEVA, SWITZERLAND pages 919 - 927 S. REBER ET AL. 'Retardation of metastatic tumor growth after immunization with metastasis-specific monoclonal antibodies.' see the whole document ---	1-12

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents:

- *'A' document defining the general state of the art which is not considered to be of particular relevance
- *'E' earlier document but published on or after the international filing date
- *'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *'O' document referring to an oral disclosure, use, exhibition or other means
- *'P' document published prior to the international filing date but later than the priority date claimed

- *'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *'&' document member of the same patent family

Date of the actual completion of the international search

12 September 1994

Date of mailing of the international search report

26-09-1994

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentiaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

Int'l	onl	ication No
PCT/FI 94/00264		

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>THE JOURNAL OF CELL BIOLOGY vol. 122, no. 2 , July 1993 , NEW YORK NY, USA pages 431 - 442 M. SALMI ET AL. 'Regulated expression of exon v6 containing isoforms of CD44 in man: Downregulation during malignant transformation of tumors of squamocellular origin.' see the whole document ---</p>	1-7,11, 12
P,X	<p>JOURNAL OF NEUROPATHOLOGY AND EXPERIMENTAL NEUROLOGY vol. 53, no. 3 , May 1994 , NEW YORK NY, USA pages 295 - 302 R. AHO ET AL. 'CD44-hyaluronate interaction mediates in vitro lymphocyte binding to the white matter of the central nervous system.' see the whole document ---</p>	1-12
P,X	<p>BLOOD vol. 84, no. 1 , 1994 , NEW YORK NY, USA pages 238 - 243 R. RISTAMÄKI ET AL. 'Serum CD44 in malignant lymphoma: An association with treatment response.' see the whole document -----</p>	1-3,11, 12